Virology 471-473 (2014) 1–12

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Sulfonation pathway inhibitors block reactivation of latent HIV-1

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ARTICLE INFO

Article history:
Received 23 May 2014
Returned to author for revisions
16 June 2014
Accepted 18 August 2014
Available online 11 October 2014

Keywords:
HIV-1
Latency
Reactivation
Primary CD4+ T cells
Gene expression
Sulfonation
Inhibitor

ABSTRACT

Long-lived pools of latently infected cells are a significant barrier to the development of a cure for HIV-1 infection. A better understanding of the mechanisms of reactivation from latency is needed to facilitate the development of novel therapies that address this problem. Here we show that chemical inhibitors of the sulfonation pathway prevent virus reactivation, both in latently infected J-Lat and U1 cell lines and in a primary human CD4+ T cell model of latency. In each of these models, sulfonation inhibitors decreased transcription initiation from the HIV-1 promoter. These inhibitors block transcription initiation at a step that lies downstream of nucleosome remodeling and affects RNA polymerase II recruitment to the viral promoter. These results suggest that the sulfonation pathway acts by a novel mechanism to regulate efficient virus transcription initiation during reactivation from latency, and further that augmentation of this pathway could be therapeutically useful.

Introduction

The development of highly active antiretroviral therapy (HAART) has dramatically improved the prognostic outlook for HIV-1 patients in the developed world. However, the success of this therapy is limited by latent viral reservoirs that persist during therapy and reseed infection if treatment is interrupted (Chun et al., 2000; Davey et al., 1999; Imamichi et al., 2001). Early estimates predicted that these reservoirs would eventually diminish during a prolonged treatment, but it is now clear that latent reservoirs will persist throughout the lifetime of most patients under the current treatment regimen (Finzi et al., 1999; Siliciano et al., 2003). This necessitates continuous therapy and creates several problems, including high cost, poor adherence, and drug resistance. Even in adherent patients, chronic exposure to both latent virus production and antiretrovirals appears to increase the risk of developing non-AIDS defining illnesses such as cardiovascular disease, diabetes, liver disease, and cancer (Bedimo, 2008; Samaras, 2009; Weber et al., 2006). For these reasons, one of the major goals of HIV-1 antiretroviral research is to develop a therapy that targets latently infected cells to facilitate drug-free remission of disease (Richman et al., 2009). Achieving this goal will require a more complete understanding of the mechanisms governing latency and virus reactivation so that novel approaches can be developed that specifically target viral reservoirs.

Viral reservoirs that persist in HAART-treated patients typically consist of long-lived cells that carry integrated proviral DNA (Pierson et al., 2000). Monocytes and macrophages have been suggested to serve as latent reservoirs because they are resistant to the cytopathic effects of HIV-1 infection. These cells can also disseminate virus to immunologically privileged sites such as the brain, where they can endure for months or even years (Cosenza et al., 2002; Gartner et al., 1986; Lassmann et al., 1993; Williams et al., 2001).

The best-characterized viral reservoir exists in resting CD4+ T cells, which typically carry markers characteristic of memory cells (Brenchley et al., 2004; Chun et al., 1997; Finzi et al., 1997;

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http://dx.doi.org/10.1016/j.virol.2014.08.016
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Wong et al., 1997). These cells can either become infected when they are activated and survive contraction to become infected memory cells or they can become directly infected while in a resting state (Cameron et al., 2010; Han et al., 2007; Jordan et al., 2003; Spina et al., 1995). Because they are not actively producing virus, infected memory $CD4^+$ T cells can be extremely long-lived. Upon activation, these cells are also capable of rapidly expanding and reseeding infection during treatment interruption (Siliciano et al., 2003). The combination of longevity and lack of actively replicating virus makes them difficult to eliminate with current therapies. Recent evidence suggests that patients that can control HIV infection in the absence of drug treatment are more likely to have unusually low levels of latent virus in long-lived CD4+ T cell subsets (Saez-Cirion et al., 2013).

Initially, mechanisms that govern HIV latency in CD4+ T cells were characterized using established cell line-based models of virus latency. Generally, these mechanisms reduce the efficiency of proviral transcription. The site of integration is partly responsible for this transcriptional suppression. In latently infected cells, the provirus tends to reside either in compacted heterochromatic regions or in very highly expressed genes that cause transcriptional interference (Han et al., 2004; Lenasi et al., 2008; Lewinski et al., 2005). Low transcriptional levels during latency can also result from decreased availability or activity of transcriptional factors that are dependent on T cell activation. Similarly, resting T cells have increased activity of repressors that drive chromatin condensation through recruitment of histone deacetylases (HDACs) (Coull et al., 2000; Hsia and Shi, 2002; Imai and Okamoto, 2006; Jiang et al., 2007; Marban et al., 2007; Tyagi and Karn, 2007; Williams and Greene, 2007).

Activation of $CD4^+$ T cells increases the availability of NF-κB, NFAT, and AP-1, leading to recruitment of co-activators to the HIV-1 LTR (Williams and Greene, 2007). The associated co-activators have histone acetyltransferase activity that cooperates with the ATP-dependent chromatin remodeling complex to displace nuc-1, the nucleosome that masks the transcription start site in the HIV-1 LTR during latency (Verdin et al., 1993). Nuc-1 displacement allows recruitment of the pre-initiation transcription complex and active viral mRNA production. The viral regulatory Tat protein increases the efficiency of this process by promoting recruitment of the positive transcriptional elongation factor (P-TEFb) and the SWI/SNF chromatin remodeling complex (Williams and Greene, 2007).

These results indicate that there are several obstacles to efficient LTR-based transcription in latently infected $CD4^+$ T cells and that the alleviation of any one of these mechanisms can activate viral replication in at least a subset of these cells. The development of successful strategies for eliminating viral reservoirs will likely require a combinatorial approach in which several of the dominant mechanisms governing latency are targeted. Indeed, several studies suggest that combinations of activating stimuli are often much more effective than individual compounds at inducing HIV reactivation in cellular models of latency (Bartholomeeusen et al., 2012; Boehm et al., 2013; Burnett et al., 2010; Perez et al., 2010; Reuse et al., 2009; Zhu et al., 2012).

We have previously shown that the cellular sulfonation pathway plays an important role in regulating HIV-1 gene expression (Bruce et al., 2008). Sulfonation is the transfer of a sulfonate group (SO_3^{-1}) from the universal donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS), generated by PAPS synthases (PAPSS1 or PAPSS2), to a hydroxyl or amino-group on an acceptor molecule. Our forward genetic screen revealed that the PAPS synthases were important for expression from the viral promoter of some (HIV-1 and murine leukemia virus), but not all (avian sarcoma and leucosis virus) retroviruses (Bruce et al., 2008). These effects were phenocopied with two chemical inhibitors of the sulfonation pathway, chlorate and guaiacol (Bruce et al., 2008; Hortin et al., 1988). These studies also indicated that these sulfonation inhibitors specifically influence de novo virus gene expression since they were effective at reducing provirus transcription immediately following virus infection but had no effect when added to cells that already contained actively expressed proviral DNA (Bruce et al., 2008).

In the present study, we have used these well-characterized inhibitors to study the role of sulfonation in latency models where sulfonation was otherwise difficult to disrupt. We show that these sulfonation inhibitors block efficient virus reactivation in established cell line models and in a primary CD4+ T-cell model of virus latency and that they can act by preventing efficient recruitment of RNA polymerase (pol) II to the virus promoter.

Results

Reactivation of the HIV-1 LTR is blocked by sulfonation inhibitors

To explore the role of sulfonation in reactivation from HIV-1 latency that has been established by different mechanisms, we used three types of well characterized latently infected cell lines: full length J-Lat clones, minigenome J-Lat clones, and promonocytic U1 cells.

The J-Lat 6.3 and 9.2 cells are clonal lines that contain transcriptionally silent full length HIV-1 provirus in which the *Env* gene is interrupted and nef is replaced with GFP to allow monitoring of reactivation by a fluorescence-based assay (Jordan et al., 2003). In J-Lat 6.3 cells, NF-κB p50 homodimers promote HDAC1 binding to the viral promoter and create a closed chromatin conformation that silences LTR expression (Williams et al., 2006). In J-Lat 9.2 cells, the latent provirus is integrated into the highly expressed *PP5* gene. *PP5* transcription causes transcriptional interference that blocks expression of the proviral genome (Lenasi et al., 2008). In both of these cell lines, DNA methylation within CpG islands in the viral promoter contributes to transcriptional silencing (Blazkova et al., 2009). Treatment with chlorate during reactivation with TNF-α alone or in combination with the HDAC inhibitor trichostatin A (TSA) decreased the degree of reactivation in J-Lat 6.3 cells by 2.7 and 9-fold (Fig. 1A and B). Similarly, in J-Lat 9.2 cells, treatment with chlorate decreased TNF-α-mediated reactivation by 2-fold and TNF-α plus TSA-mediated reactivation by 6-fold (Fig. 1A and B). Under these conditions, chlorate treatment inhibited sulfonation as expected (judged by reduced cell surface sulfotyrosine levels in J-Lat 6.3 cells) (Fig. 1D) and it did not cause any significant changes in cell viability (Fig. 1C). These data suggest that the sulfonation pathway can regulate reactivation from latency in cells where that latent state was originally maintained by NF-κB p50 homodimers or transcriptional interference.

To determine both the contribution of integration sites as well as viral proteins to the effect of sulfonation on reactivation, we next analyzed the minigenome J-Lat model cell lines. These cells contain a minimal HIV construct in which the HIV-1 LTR drives expression of GFP and Tat in the absence of additional viral proteins (Jordan et al., 2003). In J-Lat A1 cells, this construct is integrated into an intergenic region at position ChXp21.1 (Jordan et al., 2003). In J-Lat A2 cells, transcriptional interference drives latency due to integration into the highly expressed *UTX* gene (Gallastegui et al., 2011). In these cells, chromatin reassembly factors such as Spt6 can reestablish a repressive chromatin environment in the wake of RNA pol II, likely through coordination with histone deacetylases (Gallastegui et al., 2011). In J-Lat A7 cells, the construct is integrated into alphoid repetitive DNA, a component of the centromeric heterochromatin. In each of these models, sulfonation inhibition decreased reactivation triggered

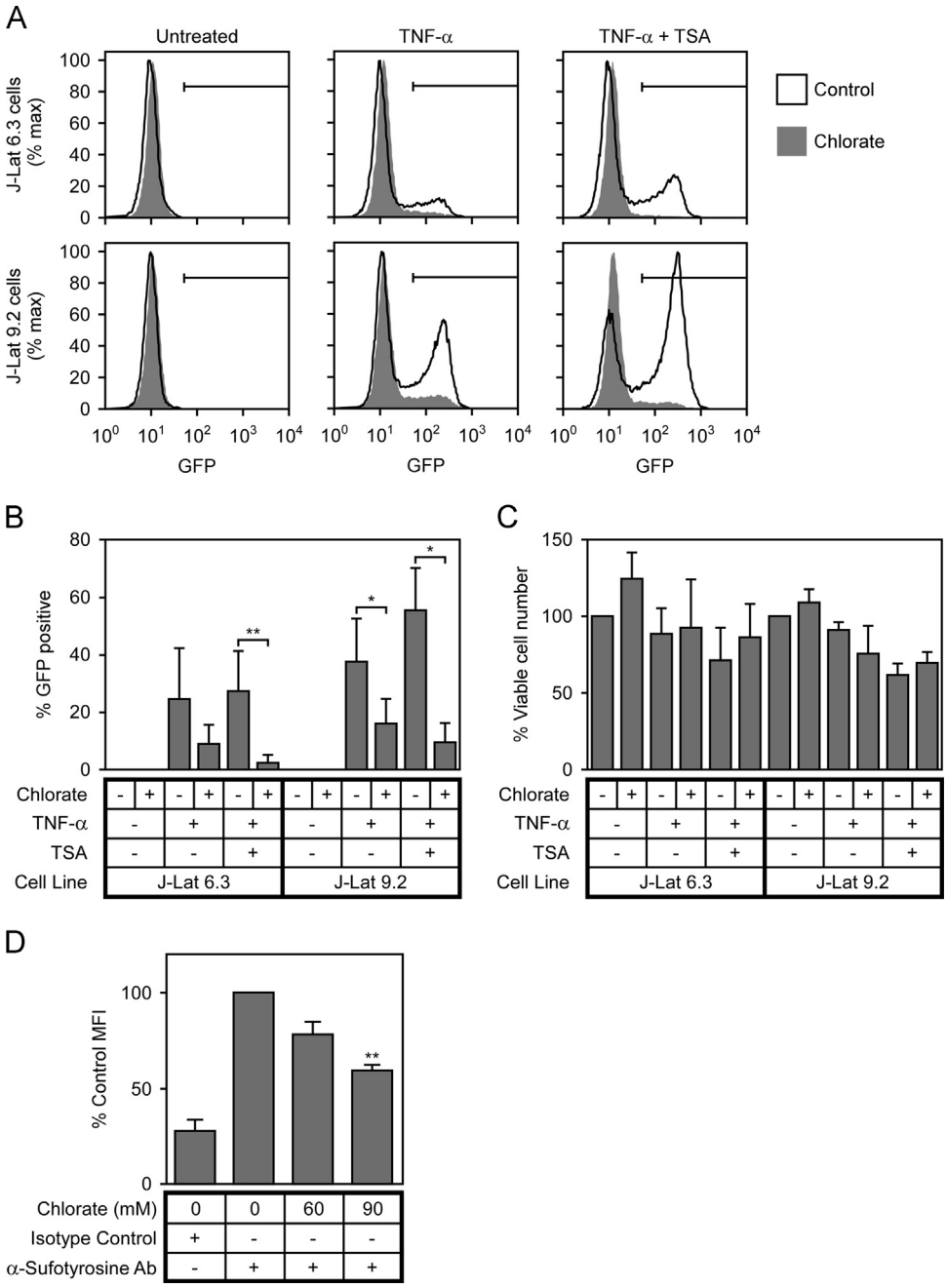

Fig. 1. Sulfonation inhibitors block HIV-1 reactivation from latency in J-Lat cells. (A) Flow cytometric analysis of latent HIV-1 reactivation in the indicated J-Lat cells lines that were unactivated or activated for 16 h with 20 ng/ml TNF-α ± 200 nM TSA in the presence (shaded curve) or absence of 90 mM chlorate (open solid curve). Histograms indicate GFP fluorescence. Gates indicate GFP-positive cells. (B, C) Bar graph representation shows the mean average values for at least three independent experiments, conducted as described in (A). The cells were assayed for GFP fluorescence using flow cytometry (B) or for viable cell number using a chemiluminescent assay (C). Error bars indicate standard deviation and statistical significance was determined by paired Student's *t*-test, $^{*}p < 0.05$, and $^{**}p < 0.01$. (D) J-Lat 6.3 cells were treated for 16 h with the indicated concentration of chlorate, then stained with anti-sulfotyrosine antibody or isotype-matched control antibody and propidium iodide. Flow cytometry was used to quantify sulfotyrosine levels on live cells, shown as mean fluorescence intensity (MFI). Mean and SEM are shown from three replicate experiments. Results are normalized to control cells in which chlorate was absent. Statistical significance was determined by one sample *t*-test, $^{**}p < 0.01$.

with TNF-α and TSA (Fig. 2A and B). While the overall degree of inhibition varied in different clonal cell lines, the inhibition of reactivation by chlorate was consistent for all the J-Lat models tested. Taken together, these results indicate that sulfonation inhibitors block reactivation in the context of multiple different mechanisms that drive viral latency in the J-Lat model system and that this effect is independent of viral proteins with the possible exception of Tat.

Several lines of evidence indicate that restricted levels of Tat lead to blocks in transcription elongation that can drive HIV-1 latency (Lassen et al., 2004; Taube and Peterlin, 2013). We used the promonocytic U1 cell line to assess the role of sulfonation in the context of latency that is induced by elongation defects. U1 cells contain two proviral HIV-1 genomes, one with a mutation that prevents Tat production and the other with a mutation that decreases Tat activity (Emiliani et al., 1998). U1 cells were activated overnight and simultaneously treated with sulfonation inhibitors. The production of intracellular p24 was used to monitor virus reactivation (Fig. 3). Either chlorate or the combination of chlorate and guaiacol inhibited reactivation with the protein kinase C

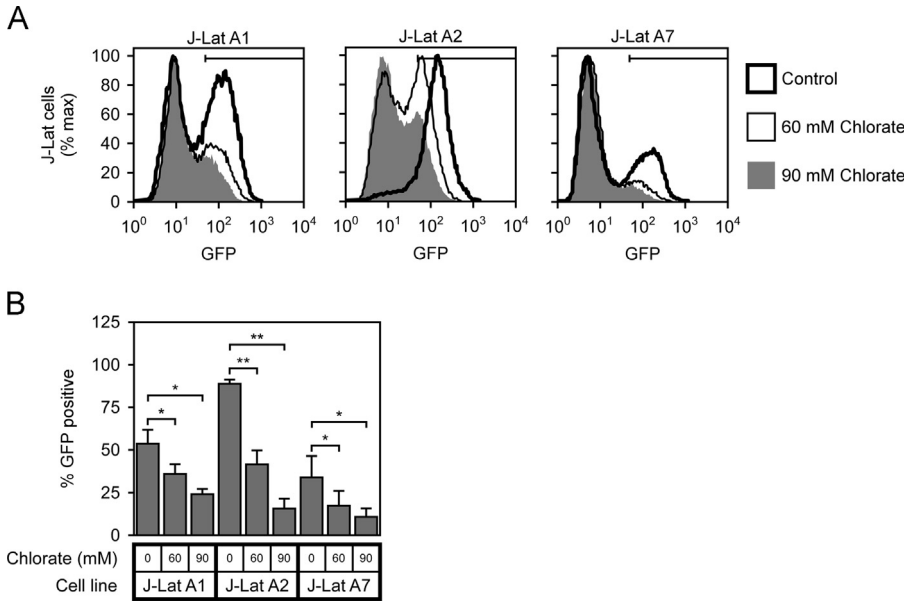

Fig. 2. Sulfonation inhibitors block reactivation from latency in J-Lat cells containing HIV minigenomes. (A) Flow cytometric analysis of latent HIV-1 reactivation in the indicated J-Lat cell lines that were activated for 16 h with 20 ng/ml TNF-α+200 nM TSA and treated without chlorate (open, thick curve), with 60 mM chlorate (open, thin curve) or with 90 mM chlorate (shaded curve). Histograms indicate GFP fluorescence. Gates indicate GFP-positive cells. (B) Bar graph representation shows the mean average values for at least three independent experiments, conducted as described in (A). Error bars indicate standard deviation and statistical significance was determined by paired Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

activator prostratin (Pro) (10-fold and 26-fold, respectively), TNF-α (3-fold and 11-fold, respectively), prostratin with TSA (4-fold and 6-fold, respectively), or TNF-α with TSA (9-fold and 10-fold, respectively) (Fig. 3A). In each of these cases, neither chlorate nor the combination of chlorate and guaiacol significantly decreased cell viability (Fig. 3B). These data indicate that the sulfonation pathway is important for reactivation in the context of latency that is maintained by defects in elongation. Thus, in several established models for HIV latency in which different mechanisms have been shown to maintain the latent state, sulfonation pathway inhibitors consistently block reactivation with multiple stimuli.

Sulfonation pathway inhibitors reduce the level of initiated HIV-1 transcripts following reactivation

To determine if the sulfonation pathway inhibitors impact HIV-1 transcription initiation or elongation, we performed a quantitative RT-PCR analysis of viral RNA with 2 different oligonucleotide primer sets (Fig. 4A). The first, designated as the "initiated" set amplifies the 5′-end of the viral transcript (residues +1 to +59), a region that contains the viral TAR element, which is under Tat-dependent transcriptional elongation control (Adams et al., 1999; Lassen et al., 2004). These "initiated" primers will amplify all transcripts that are successfully initiated, both abortive transcripts and those for which elongation is successfully completed. The second pair of oligonucleotide primers, designated as the "elongated" set, was used to amplify a downstream region (residues +107 to +189), which is beyond the two major pause sites within the HIV LTR at +50 and +119 (Jadlowsky et al., 2014). As a result, this primer set only amplifies transcripts in which HIV-1 Tat has facilitated transcriptional elongation (Ott et al., 2011). In J-Lat 6.3 cells, chlorate treatment significantly reduced the levels of both types of transcript at early times post-activation (Fig. 4B and C). Within 4 h of activation, there was a 2-fold reduction in initiated transcripts and a 9-fold reduction in elongated transcripts. The greater effect on elongated transcripts in these cell lines might result from the low level of transcription initiation that takes place during latency in the absence of sufficient Tat production. Within 6 h of activation, initiated and elongated transcripts were reduced 12-fold and 20-fold, respectively. Primers that amplified a transcript in the vpr gene showed sulfonation-dependent decreases in HIV transcription that mirrored those seen with the initiated and elongated primer sets (data not shown). Treatment of U1 cells with chlorate, guaiacol, or the combination of both inhibitors led to a similar decrease in both initiated and elongated HIV-1 transcripts (Fig. 4D and E). These data are consistent with a model in which sulfonation pathway inhibitors act at the level of transcription initiation.

Sulfonation does not broadly alter the expression of TNF-α and TSA-responsive genes in Jurkat cells

The HIV-1 LTR promoter is strongly affected by transcription factors that are upregulated during T cell activation (Williams and Greene, 2007). Thus, it is possible that sulfonation blocks TNF-mediated reactivation from latency by broadly disrupting TNF-α and TSA-mediated signaling pathways. To address this question, J-Lat 6.3 cells were treated with TNF-α and TSA in the presence or absence of 90 mM chlorate for 6 h, then cellular RNA was extracted and subjected to microarray analysis. Under these conditions, treatment with TNF-α and TSA significantly changed the signal of 462 probe sets, specific for 392 genes (Table S1). These genes were most strongly enriched for a cluster of functional categories associated with immune system development, lymphocyte activation, and apoptosis (Table S2). However, only 22 of these genes (25 probe sets) were affected by the treatment with the sulfonation inhibitor (Table S3) and just 2 of the genes affected by sulfonation (CD24, DHRS2), were annotated in the lymphocyte activation cluster. These results indicate that the sulfonation pathway selectively regulates TNF-α and TSA-induced HIV-1 gene expression.

LTR regulation by sulfonation is independent of NF-κB

Several transcription factors are known to bind to defined sites within the HIV-1 LTR. To determine if these binding sites were required for regulation by sulfonation, we used a panel of DHIV

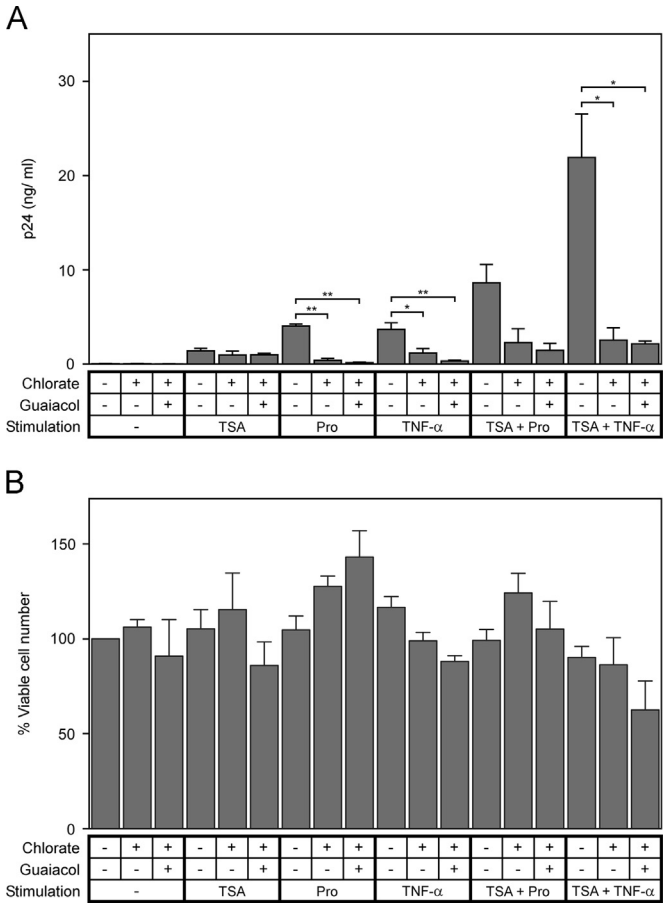

Fig. 3. Sulfonation inhibitors block reactivation from latency in U1 cells. (A) U1 cells were treated for 16 h with 400 nM TSA, 5 μM Prostratin, 10 ng/ml TNF-α, 200 nM TSA and 5 μM prostratin, or 200 nM TSA and 10 ng/ml TNF-α. At the time of activation, cells were treated with 60 mM chlorate or 60 mM chlorate and 2 mM guaiacol. After activation for 16 h, cellular lysates were assayed by ELISA for p24 levels (A) and the viable cell number was assessed using a chemiluminescent assay (B). The mean values from three independent experiments performed in triplicate are shown. Error bars indicate standard error. Statistical significance was determined by paired Student's *t*-test, $^{*}p < 0.05$, $^{**}p < 0.01$.

viruses containing defined LTR mutations that disrupt the binding sites for NF-kB, NFAT, NF-IL6, and USF (Bosque and Planelles, 2009). These viruses were used to infect HOS-CD4 cells in the presence or absence of sulfonation inhibitors, then virus infection was quantified using qRT-PCR to measure HIV mRNA transcription. Sulfonation inhibitors decreased transcription from each mutant virus to the same degree as that seen with the wild type virus (Fig. 5). This shows that these transcription factors are not required for sulfonation-mediated regulation of LTR transcription and suggests that sulfonation regulates a step in transcription that occurs after these transcription factors bind the HIV-1 LTR.

Sulfonation inhibitors do not influence chromatin remodeling at the viral promoter

Transcription initiation of the latent provirus is regulated by nucleosome positioning. In the latent state, the HIV-1 LTR exists in a closed conformation in which the transcription start site is masked by the nuc-1 nucleosome (Verdin et al., 1993). During reactivation from latency this nucleosome is remodeled, making the start site more accessible to the core RNA pol II transcription machinery. This alteration can be monitored by AflII restriction enzyme site accessibility (Fig. 6A) (Verdin et al., 1993).

To determine if sulfonation inhibitors influence nuc-1 remodeling, nuclei were extracted from cells mock-treated or treated with several different activators. The nuclei were digested with AflII before DNA was purified and digested to completion with PstI, which cuts upstream and downstream of the viral LTR (Fig. 6A). The digested samples were then analyzed by Southern blotting using a viral probe corresponding to a region downstream of the LTR U5 region (Fig. 6A). As expected, AflII cleaved the provirus promoter region in activated cells (Fragment a, Fig. 6B, lanes 3, 5, 7, and 9) but not in unactivated U1 cells (Fig. 6B, lane 1). Individual chlorate and guaiacol treatment or treatment with both inhibitors did not abolish nuc-1 remodeling during reactivation from latency with different stimuli (Fig. 6B lanes 4, 6, 8, and 10 and Fig. 6C lanes 6–8). Instead, these treatments appear to promote this rearrangement, even in the absence of an activating stimulus (Fig. 6B lane 2 and Fig. 6C lanes 2–4). The combination of chlorate and guaiacol treatments gave rise to stronger nuc-1 remodeling than either inhibitor alone (Fig. 6C, compare lanes 2–4). This suggests that inhibition of reactivation could result from erroneous nucleosome remodeling or that the effect of the sulfonation inhibitors on transcription initiation takes place at a step downstream of nuc-1 remodeling.

Sulfonation inhibitors reduce RNA pol II recruitment to the HIV LTR

Chromatin immunoprecipitation (ChIP) studies were employed to test the effect of sulfonation inhibitors on RNA pol II recruitment to the HIV-1 LTR. J-Lat 6.3 cells were activated with TNF-α and TSA, protein-DNA complexes were crosslinked and the samples subjected to immunopurification using an antibody specific for RNA pol II. The level of RNA pol II associated with the viral promoter was then determined by qPCR analysis of proviral DNA.

We found that the overall levels of RNA pol II associated with the viral promoter increased by 5.5-fold during activation with TNF-α and TSA ($p=0.020$) (Fig. 7A). By contrast, chlorate reduced the level of RNA pol II associated with the promoter 1.9-fold ($p=0.0040$) (Fig. 7A). Even with chlorate present, there was still a 2.8-fold increase in RNA pol II levels relative to the untreated controls, though this was not significant ($p=0.13$). This residual recruitment could be due to the incomplete inhibition of transcription by chlorate or it could indicate that chlorate affects promoter-proximal pausing and clearance as well as RNA pol II recruitment. The chlorate treatment did not significantly alter the levels of RNA pol II at the cellular GAPDH promoter used for control purposes, indicating that the effect of the inhibitor was specific for the viral promoter (Fig. 7B). Also, the viral promoter was not enriched in parallel ChIP experiments performed with a control IgG (Fig. 7A) and a non-transcribed DNA fragment was not co-purified with the pol II antibody (Fig. 7C). We conclude that sulfonation inhibitors block HIV-1 gene expression in these cells at least in part by decreasing RNA pol II recruitment to the HIV-1 LTR promoter.

Sulfonation inhibitors block transcription initiation during reactivation in a primary CD4 T-cell model of virus latency

To assess the effects of sulfonation inhibition in a more physiologically relevant cell system, we used an in vitro primary CD4+ T-cell model of HIV latency. In this model, non-productive viral infection is established in a non-dividing subpopulation of CD4+ T cells isolated after co-culture with activated autologous infected cells. Importantly, latent virus can be reactivated in this model system by several physiologically relevant inducers, including HDAC inhibitors, PKC agonists, TNF-α, and TCR engagement (Spina et al., 2013).

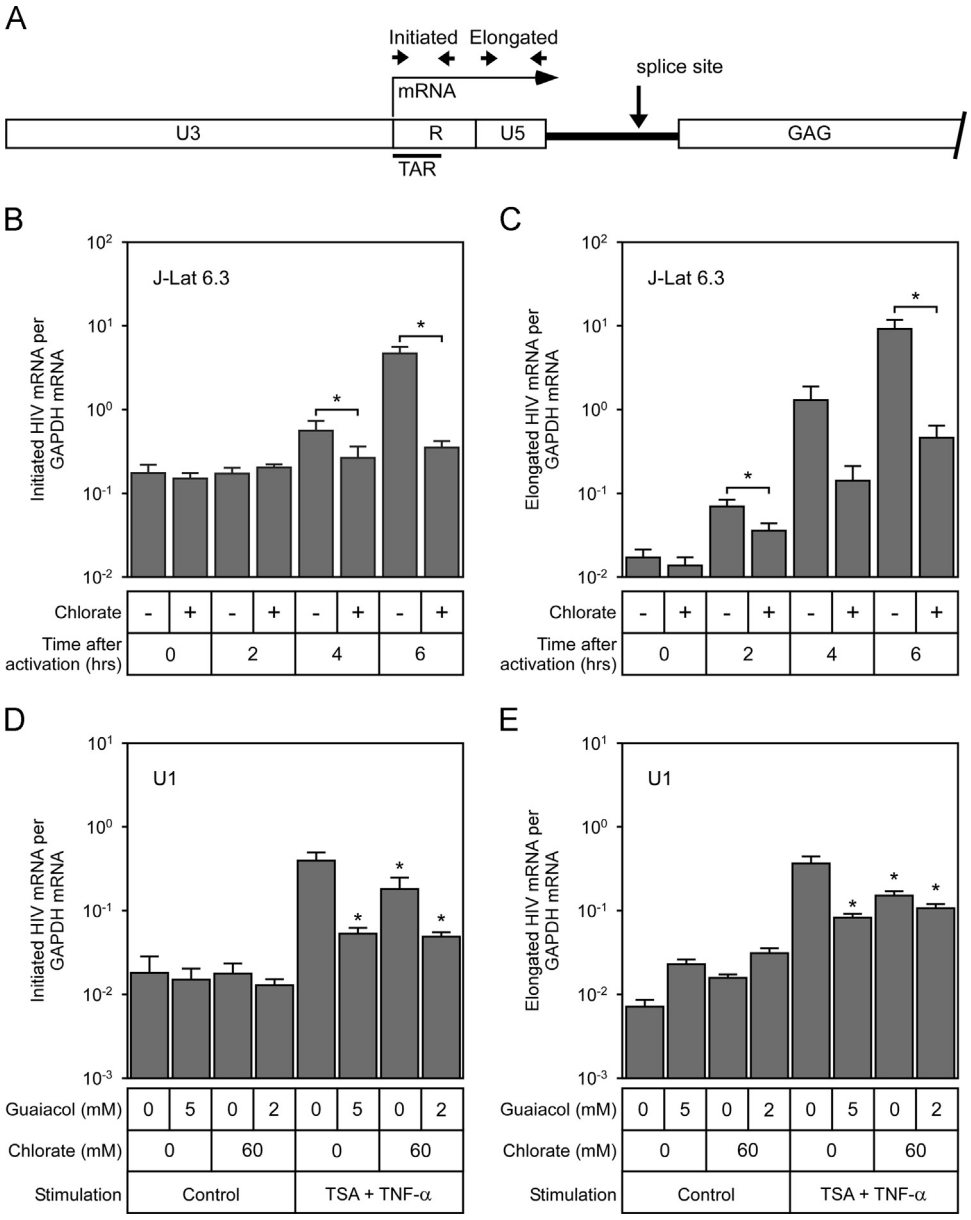

Fig. 4. Sulfonation inhibitors reduce the level of initiated HIV-1 transcripts during reactivation. (A) Diagram of the HIV-1 LTR showing the location of the "initiated" and "elongated" primer sets. (B, C) Quantitative RT-PCR measurements of initiated (B) and elongated (C) HIV-1 transcripts, normalized to a cellular GAPDH control, at different time points post-activation following treatment of J-Lat 6.3 cells with 20 ng/ml TNF-α and 200 nM TSA ± 90 mM chlorate. (D, E) U1 cells were mock treated or treated for 6 h with 20 ng/ml TNF-α and 200 nM TSA ± 5 mM guaiacol, 60 mM chlorate, or 60 mM chlorate and 2 mM guaiacol. The viral RNA species were then monitored as in panels B and C. For panels B–E, statistically significant differences from the mock treated controls were determined using the paired Student's *t*-test, $^{*}p < 0.05$. Bars indicate the mean and error bars indicate standard error of at least 4 independent experiments, each performed in triplicate.

Provirus reactivation in the latently infected resting CD4+ T cell population was stimulated by T cell receptor activation with immobilized α-CD3/α-CD28 antibodies for 48 h and was monitored by measurement of cell-associated HIV-1 Tat mRNA expression normalized to 18S rRNA levels. Guaiacol and/or chlorate treatment reduced the levels of induced HIV-1 Tat mRNA between 2 and 7-fold (Fig. 8A), without affecting the percentage of viable cells (Fig. 8E). These effects were also clearly seen when the values obtained from each experiment were normalized to the untreated activated cell control, accounting for variation between donors in the overall level of induced activation (Fig. 8B). To determine if the block to virus reactivation in this primary T cell system also maps to transcription initiation, we employed the same qRT-PCR based approach used in Fig. 4. Indeed, treatment with chlorate and guaiacol reduced the abundance of both RNA species greater than 10-fold (Fig. 8C and D), indicating that these inhibitors also block transcription initiation in this primary cell model system.

Discussion

We have shown that two independent inhibitors of the sulfonation pathway, chlorate and guaiacol, can block efficient HIV-1 reactivation in established cell lines and in a primary CD4+ T-cell model system of virus latency. This effect is likely to be broad-acting since previous studies in the established cell line models showed that proviruses in these cells are maintained in the latent state by a variety of different mechanisms including transcriptional interference, inefficient Tat activity, repressive DNA methylation, transcription factor binding, or chromatin context.

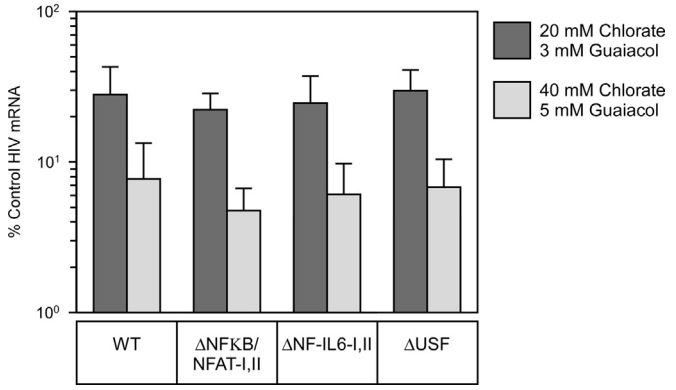

Fig. 5. NF-kB, NFAT, NF-IL6, and USF binding sites are not required for regulation by sulfonation. HOS-CD4 cells were infected with wt DHIV or with different LTR mutants (Bosque and Planelles, 2009) in the presence of 40 mM chlorate and 5 mM guaiacol (light gray bars), 20 mM chlorate and 3 mM guaiacol (dark gray bars), or no drug (normalized to 100% infection). 30 h after infection, RNA was extracted and viral mRNA was quantified relative to cellular actin mRNA. Bars indicate the mean of at least 3 independent experiments, each performed in triplicate.

Sulfonation inhibition decreases both initiated and elongated HIV transcripts, indicating that sulfonation decreases transcription initiation. We also provided evidence that these inhibitors can act downstream of nucleosome remodeling and decrease RNA pol II recruitment to the viral promoter in the J-Lat model. By extending the regulation of the sulfonation pathway to reactivation of virus gene expression, these results support our earlier conclusion, based on genetic and pharmacological evidence, that sulfonation can regulate de novo HIV-1 gene expression (Bruce et al., 2008). Thus, these data combined with our earlier findings (Bruce et al., 2008), provide two independent lines of evidence that the sulfonation pathway can play a role in regulating de novo virus gene expression, either immediately following virus infection or during reactivation from latency.

The experiments described here used chlorate and guaiacol as tools to explore the role of the sulfonation pathway in the regulation of HIV-1 latency. As described above, these compounds have been shown to inhibit expression of newly integrated retroviral DNA in a manner that phenocopies PAPS synthase deficiency (Bruce et al., 2008). We did attempt to phenocopy the effects of the chemical inhibitors on HIV-1 reactivation by expressing inducible shRNAs targeting *PAPSS1* and *PAPSS2* in J-Lat cells. However, we found that expression of any shRNA (including control shRNAs) in these cells led to a modest increase in viral mRNA even in the absence of any other added stimulus. This effect was more pronounced when multiple shRNA constructs were introduced into the same cell line. While the underlying mechanism behind these results is unknown, this effect prevented us from using genetic means to validate results obtained with pharmacological inhibitors.

Our finding that sulfonation inhibitors block RNA pol II recruitment without decreasing chromatin remodeling is surprising, as these two processes are generally closely linked. Indeed, we found that sulfonation inhibitors actually increase nuc-1 remodeling. This could be directly linked to the decrease in transcription, as in the case of the yeast protein Fun30, which represses transcription by altering nucleosome positioning (Byeon et al., 2013). Alternatively, transcriptional repression could be independent of nucleosome remodeling if sulfonation inhibitors block a step that occurs downstream of this process.

Typically, during HIV-1 reactivation from latency, nucleosomal remodeling is associated with increased histone acetylation and recruitment of RNA pol II (Van Lint et al., 1996; Williams et al., 2006). However there are examples in which histone acetylation takes place without chromatin remodeling (Kiefer et al., 2004) and

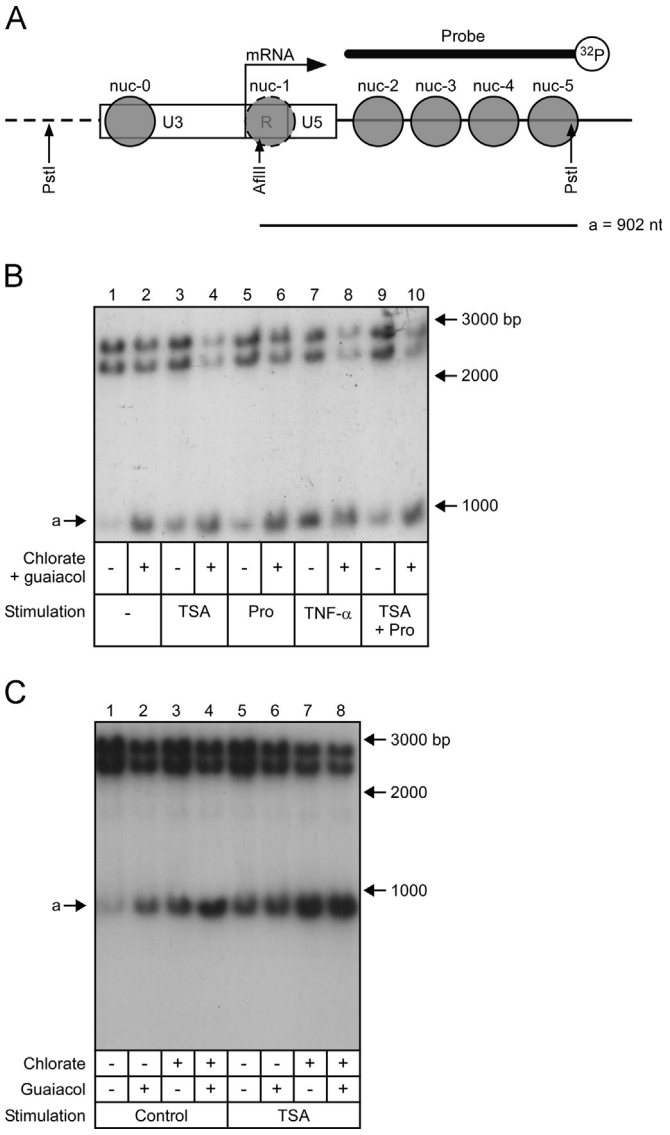

Fig. 6. Sulfonation inhibitors do not block nuc-1 remodeling. (A) Diagram shows the positions of nucleosomes bound to the HIV-1 LTR and the location of the AflII and PstI restriction enzyme sites (Verdin et al., 1993). PstI cleaves the HIV-1 genome at the indicated site and at a site within the host genomic DNA located upstream of the integrated provirus. The size of the fragments generated by partial digestion with AflII, followed by complete digestion with PstI, is shown. (B) Nuclei were prepared from U1 cells mock treated or treated with 400 nM TSA, 5 μM prostratin, 10 ng/ml TNF-α, or 200 nM TSA and 5 μM prostratin in the presence or absence of 60 mM chlorate and 2 mM guaiacol for 60 min and partially digested with AflII. (C) Nuclei were prepared from U1 cells mock treated or treated with 400 nM TSA in the presence or absence of 5 mM guaiacol, 60 mM chlorate or 60 mM chlorate and 2 mM guaiacol for 60 min and partially digested with AflII. After partial digestion, DNA was purified, digested to completion with PstI, and then analyzed by Southern blot analysis using a probe corresponding to a region located downstream of the LTR U5 region (A). The fragment that results from cleavage with AflII is indicated (a). Bands located between 2000 and 3000 bp result from cleavage of genomic DNA upstream of the proviruses. Nucleic acid molecular size markers are indicated.

in which chromatin remodeling takes place in the absence of histone acetylation (Klichko et al., 2006). Our studies have pinpointed a novel regulatory step in HIV-1 gene expression, one that maps after chromatin remodeling and prior to RNA pol II recruitment. This distinguishes the HIV-1 LTR from promoters such as the α_1-antitrypsin promoter, which is stimulated by chromatin remodeling that occurs after the RNA pol II preinitiation complex is fully assembled (Soutoglou and Talianidis, 2002).

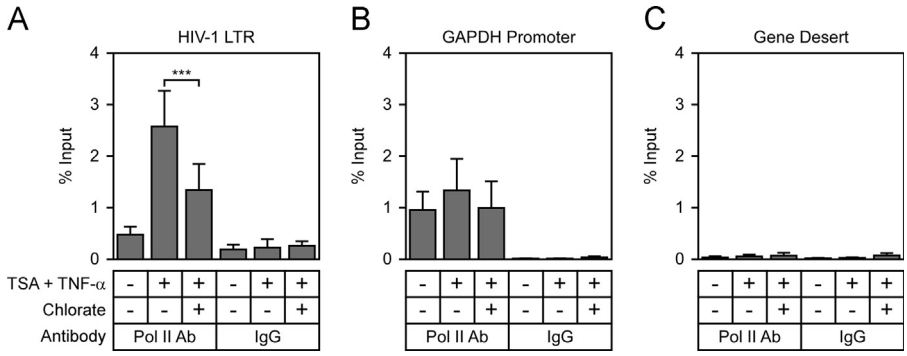

Fig. 7. Sulfonation inhibitors block RNA pol II recruitment to the HIV-1 LTR. Chromatin immunoprecipitation analysis of the HIV-1 proviral LTR in J-Lat 6.3 cells in the absence of treatment, in the presence of 20 ng/ml TNF-α and 200 nM TSA, or in the presence of 20 ng/ml TNF-α, 200 nM TSA, and 90 mM chlorate. After 6 h, cells were lysed and sonicated. Samples were immunoprecipitated with an antibody against RNA pol II or with non-specific IgG, then probed by qPCR using primers specific for the HIV-1 LTR (A), the GAPDH promoter (B), or a region of chromosome 12 that contains no active genes (C). All graphs represent mean and standard error of at least 4 independent experiments performed in triplicate. Where indicated, statistical significance was determined using the paired Student's *t*-test, $^{***}p < 0.005$.

Studies on transcription regulation in several different systems have led to a model in which activators such as NF-κB or NFAT recruit chromatin modifiers and remodelers, which allow the subsequent formation of the RNA pol II preinitiation complex (Malik and Roeder, 2010; Siliciano and Greene, 2011). Preinitiation complex formation is regulated by several factors, including the mediator complex and general transcription factors (Sikorski and Buratowski, 2009). For example, the mediator complex can bind the histone acetyltransferase p300, causing p300 dissociation from the promoter and facilitating the binding of TFIID and other components of the preinitiation complex (Black et al., 2006). Our data support a model in which sulfonation inhibitors block the function of one of the factors mediating preinitiation complex assembly. Further experiments are needed to determine the effects of the sulfonation pathway on preinitiation complex assembly at the HIV-1 LTR.

The primary T cell model of HIV-1 latency we employed used direct infection of resting CD4+ T cells, which represent bystanders in an ongoing productive infection of proliferating T cells. This is a relevant model system for studying HIV-1 reactivation, one that recapitulates a number of features seen with clinical samples (Spina et al., 2013). However, each primary cell model system has its own merits and the clinical features of HIV-1 reactivation are not recapitulated exactly by any single model system. Indeed, simply removing latently infected cells from ART-treated patients and culturing them ex vivo likely alters their responsiveness to certain stimuli (Spina et al., 2013). The differences between model systems is demonstrated in that sulfonation inhibitors did not block HIV-1 reactivation in a non-polarized central memory CD4+ T cell model (Bosque and Planelles, 2009) (data not shown). Similarly, HDAC inhibitors were inactive in this non-polarized central memory model, but active in the model based on bystander spread (Spina et al., 2013). Further studies will be needed to determine if the sulfonation pathway controls HIV-1 reactivation in other latency models.

In addition, it will be important to determine whether one or more cytosolic or golgi cellular sulfotransferases—enzymes that use PAPS to transfer sulfonate to a target molecule (Strott, 2002)—control HIV-1 gene expression. Once the specific sulfotransferase (s) involved has been identified, it should become possible to screen for compounds that augment their activity which, in turn, would be predicted to promote more efficient reactivation from latency. In this way, the sulfonation pathway may provide a novel target that could be used, in combination with other treatments, to facilitate the development of a therapeutic strategy designed to cure HIV-1 infection during a suppressive antiretroviral treatment.

Materials and methods

Cell culture and reagents

The promonocytic U1 cell line (Folks et al., 1987) and the J-Lat 6.3, 9.2, A1, A2, and A7 lines (Jordan et al., 2003) were obtained from the AIDS Research and Reference Reagent Program (NIAID, NIH, Bethesda, MD). Cells were grown in RPMI 1640 medium (Gibco-BRL) supplemented with 10% fetal bovine serum, 50 U/ml of penicillin, 50 μg/ml streptomycin at 37 °C in a humidified 95% air/5% CO_2 atmosphere.

Cells were stimulated with 200 nM trichostatin A (TSA, Sigma-Aldrich), 5 μM prostratin (12-deoxyphorbol-13-acetate, Sigma-Aldrich), or 20 ng/ml TNF-α (Gibco-BRL) unless otherwise indicated. Sodium chlorate and guaiacol were obtained from Sigma-Aldrich. All compounds were resuspended and stored as recommended by the manufacturer, then diluted in cell culture medium immediately before use. Viable cell number was assayed using CellTiter-Glo reagent (Promega) following the manufacturer's instructions.

p24 ELISA

Production of HIV-1 p24 in U1 cells after reactivation for 16 h was measured by p24 antigen capture ELISA according to manufacturer's instructions (Zeptometrix).

Transcriptional microarray profiling

J-Lat 6.3 cells were stimulated for 6 h with 20 ng/ml TNF-α and 200 nM TSA (± 90 mM sodium chlorate). Total RNA was isolated using Qiagen RNeasy kits and hybridized to human U133A 2.0 microarrays using standard protocols (Affymetrix). Data were generated from 3 independent samples per condition. Statistical analyses were conducted using an unpaired-test version of the VAMPIRE algorithm for microarray data (Hsiao et al., 2005). Statistically significant expression changes were identified using a Bonferroni error threshold of $\alpha_{Bonf}=0.05$, which accounts for multiple-testing errors. DAVID analysis (Huang da et al., 2009a, b), using standard parameters and high stringency was conducted to identify clusters of GO categories.

Real-time quantitative PCR

For J-Lat cells, lysates from 50,000 cells were used for cDNA synthesis reactions using random oligomers according to manufacturer's instructions using the Cells-to-C_t kit (Life Technologies).

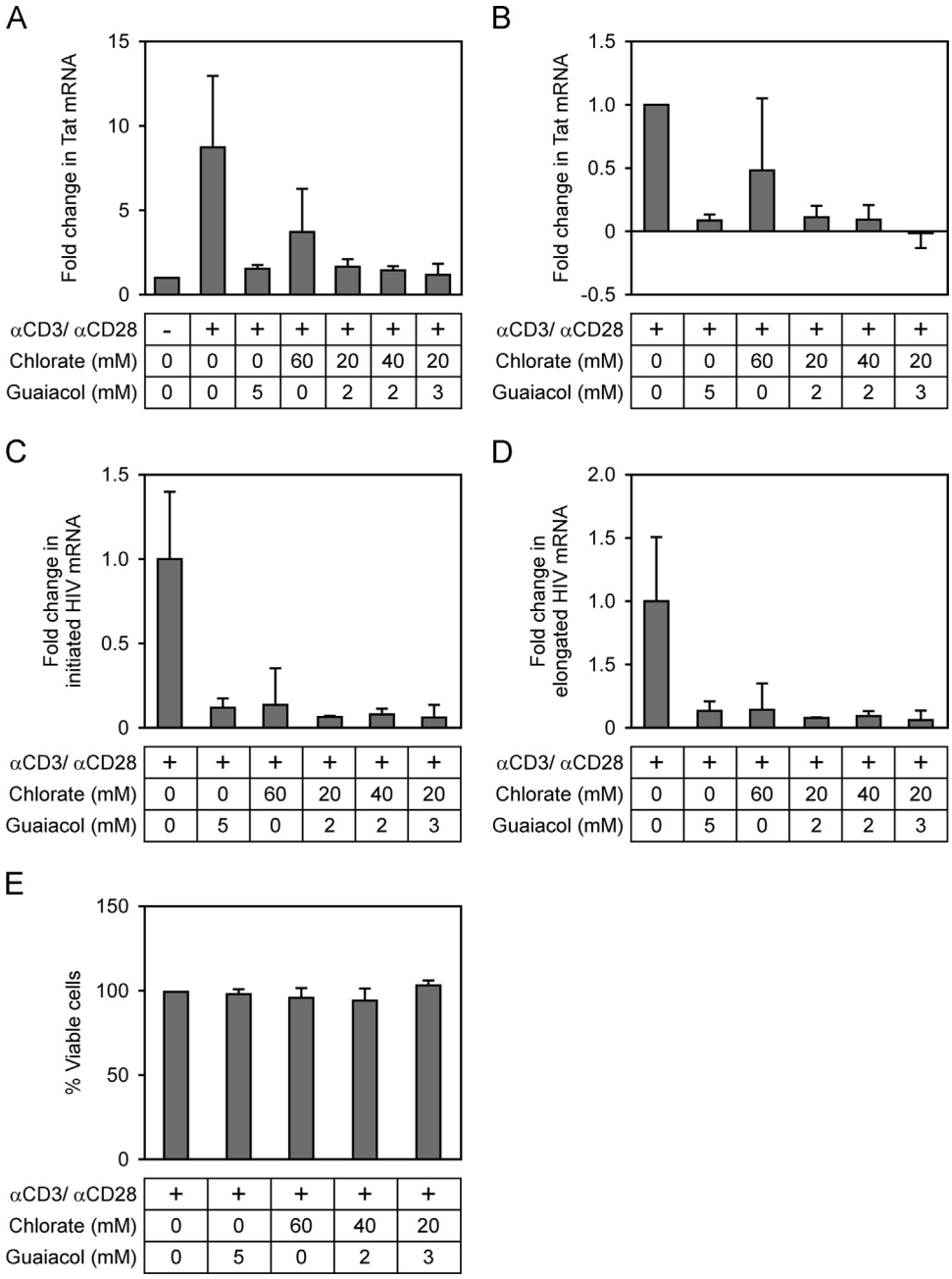

Fig. 8. Sulfonation inhibitors block transcription initiation in a primary CD4+ T-cell model of virus reactivation. Latently infected, resting CD4+ T cells were recovered from in vitro culture (as described in the Materials and Methods section) and activated for 48 h with α-CD3/α-CD28 antibodies. Chlorate or guaiacol were added at the indicated concentrations 2 h prior to initiation of cell activation and then maintained during the 48 h reactivation period. (A) Viral mRNA levels were measured by qRT-PCR, using primers specific for multiply spliced HIV Tat mRNA and normalized to a cellular 18S rRNA control. The mean average values from three experiments using different donor cells are shown. (B) Results obtained shown in (A) are shown normalized to the activated control values. (C, D) Initiated and elongated viral mRNA species were quantified using the primers shown in Fig. 4A and normalized to a cellular 18S rRNA control. The data shown represent the mean values obtained with cells from two different experiments using different donor cells. Error bars indicate standard deviation. (E) CD4+ T cells were activated in the presence of chlorate and/or guaiacol for 48 h with α-CD3/α-CD28 antibodies, stained with fixable violet dead cell stain, and fixed with paraformaldehyde. The percent of violet-negative cells was determined by flow cytometry and normalized to a control without chlorate or guaiacol. The data shown represent the mean values of three experiments and error bars indicate standard deviation.

The Cells-to-C_t kit yielded inconsistent results with U1 cells, so an alternative method was used to purify RNA and synthesize cDNA from these cells. Total RNA samples were isolated using the miRNeasy kit (Qiagen) and used to synthesize cDNA using the Quantitect reverse transcription kit (Qiagen). Real-time PCR for detection of total and elongated HIV-1 transcripts was performed using previously described primers: for initiated HIV transcripts (Adams et al., 1999; Lassen et al., 2004), HIV-START, 5′-GGGTCTCTCTGGTTAGA-3′ and HIV-SHORT, 5′-GGGTTCCCTAGTTAGCC-3′; for Elongated HIV transcripts (Konig et al., 2008), ERT2F, 5′-GTGCCCGTCTGTTGTGTGAC-3′ and ERT2R, 5′-GGCGCCACTGCTAGAGATTT-3′; for Vpr HIV transcripts, VPR1F, 5′-TGGAACAAGCCCCAGAAGACC-3′ and VPR1R, 5′-TGCCCAAGTATCCCCATAAGTTTC-3′. As an input control, a primer set that detects glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used: GAPDH-F, 5′-CATGAGAAGTATGACAACAGCCT-3′ and GAPDH-R, 5′-AGTCCTTCCACGATACCAAAGT-3′. PCR was performed by heating the reaction mixture to 95 °C for 20 s and 40 cycles of 95 °C for 1 s, 60 °C for 20 s. Amplification and detection were performed using an ABI Prism 7900HT Fast Real-Time PCR System and Fast SYBR Green PCR Master Mix (Life Technologies) in 384-well optical reaction

plates. Duplicate 10 μl reaction mixtures contained 200 nM of the Elongated or GAPDH PCR primer pairs or a 1 μM concentration of the initiated primer pair. Transcript levels were determined using standard curves prepared from plasmids containing HIV proviral DNA or GAPDH cDNA.

For infected primary CD4+ T cells, HIV tat transcripts were quantified using a modification of a published method (Fischer et al., 2002). Total cellular RNA was extracted (Qiagen RNeasy kit) from sample aliquots of 5×10^5 cells and qRT-PCR was performed with the samples, uninfected T cell RNA, and the *tat* RNA standard control (normalized to 25 ng input RNA). The isolated RNA was reverse transcribed by random hexamer priming. The cDNA products were analyzed by qPCR with primers (400 nM) directed to cellular 18S and HIV *tat*: TAT-F, 5′-GGCGACTGAATTGGGTGTC-3′ and TAT-R, 5′-TCTACTGGCTCCATTTCTTGCT-3′. Probes (250 nM) were 5′-FAM, 3′-Black Hole Quencher labeled (Integrated DNA Technologies), TAT-P, TCCTCTGTCGAGTAACGCCTATTC. The standard curve for *tat* transcript was generated by serially diluting the reverse transcribed, cloned RNA into uninfected cellular cDNA.

Flow cytometry analysis

J-Lat cells were mock-treated or treated for 16 h with 20 ng/ml TNF-α or 20 ng/ml TNF-α and 200 nM TSA in the presence or absence of the indicated concentrations of chlorate for 16 h. Cells were fixed using 1.5% formaldehyde, the percentage of live GFP-positive cells was measured on a FACScan cytometer (Becton Dickinson) and data were analyzed using FlowJo software (TreeStar). Analysis was restricted to the single cell population, as defined by forward versus side scatter profile.

To assay viability of primary CD4+ T cells, 10^6 cells were activated with α-CD3 and α-CD28 antibodies on Dynabeads to induce cell proliferation according to manufacturer's instructions (Life Sciences). Cells were treated with chlorate and/or guaiacol at the indicated concentrations for 48 h. Cells were stained according to manufacturer's instructions in Dulbecco's phosphate buffered saline, calcium- and magnesium-free (PBS, Life Technologies) with the viability dye Live/Dead violet (Life Technologies) and fixed in 1% paraformaldehyde in DPBS. The number of viable, violet-negative cells was determined on a LSRII cytometer (Becton Dickinson).

To assess sulfotyrosine levels, 10^6 J-Lat 6.3 cells were treated with the indicated levels of sodium chlorate for 16 h. Cells were washed in staining buffer (PBS with 2% FBS), resuspended in 100 μl staining buffer with 2 μl mouse anti-human sulfotyrosine antibody (Millipore, Cat. no. 05-1100) or 4 μl IgG2aκ mouse isotype control (BD Biosciences, Cat. no. 553454) and incubated at room temperature for 1.5 h in the dark. The cells were then washed and resuspended in 200 μl staining buffer with 1 μl goat anti-mouse AlexaFlour680-conjugated IgG (H+L) secondary antibody (Life Technologies) and incubated in the dark at room temperature for 30 min. Control experiments using mouse embryonic fibroblasts lacking TPST1 and TPST2 (Westmuckett et al., 2008) showed that this anti-human sulfotyrosine antibody is specific for sulfotyrosine residues under these conditions. The cells were washed, resuspended in 500 μl staining buffer with 2.5 μl 1 mg/ml propidium iodide (Life Technologies), incubated for 10 min at room temperature, washed and resuspended in staining buffer. Sulfotyrosine levels were quantified on live (propidium iodide negative) cells by flow cytometry using a BD Accuri Flow Cytometer.

Virus generation and viral infection

Plasmids containing defective HIV (DHIV) were mutagenized as described (Bosque and Planelles, 2009). Single-cycle VSV-G pseudotyped lentiviruses were generated by transient transfection of HEK293T cells (Konig et al., 2007). To normalize infections, p24 levels were determined for viral stocks using p24 ELISA. Viruses normalized by p24 were used to infect HOS-CD4 cells for 4 days, then DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen). Real time PCR was used to quantify proviral copies, determined using the ERT2F and ERT2R primers, relative to copies of genomic DNA, determined using primers ZASC1_51F, 5′-TTGGTGACTGTCATCCTGTTTC-3′ and ZASC1_51R, 5′-CTAGCACC-CATGGTTAAGGAAG-3′. 10^4 HOS-CD4 cells were infected at a MOI of 0.5 in the presence or absence of the indicated concentrations of chlorate and guaiacol. After 30 h, cells were lysed, cDNA was synthesized, and viral mRNA was quantified using the ERT2F and ERT2R primers. Virus expression was normalized to actin mRNA, using the actinF, 5′-CCTGGCACCCAGCACAAT-3′, and actinR, 5′-GCCGATCCACACGGAGTACT-3′ primers. QRT-PCR was used to determine mRNA copy number as described above.

Analysis of HIV LTR nucleosome 1 (nuc-1) chromatin structure

Purified nuclei were digested with AflII or HinfI as described elsewhere (Verdin et al., 1993). Purified DNA (30 μg) was digested to completion with PstI. The fragments were separated by electrophoresis using 1.5% agarose gels and analyzed by Southern blot. The virus promoter-specific probe was synthesized using PCR products generated using the primer set EV1 and EV2 as described (Verdin et al., 1993). These PCR products were used to generate labeled probe using [α-^{32}P] CTP and the Rediprime II DNA labeling system, then purified using Illustra ProbeQuant G-50 micro columns according to manufacturer's instructions (GE Healthcare).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described elsewhere (Barish et al., 2010). J-Lat cells were cross-linked after drug treatments. To detect chromosomal flanking regions, pellets were sonicated (Bioruptor sonicator) to obtain DNA fragments of 100–400 nt. Chromatin immunoprecipitations were performed with an antibody directed against RNA pol II (catalog no. sc-899, Santa Cruz Biotechnology). Quantitative real-time PCR reactions were performed as described above using 200 nM each primer specific for the HIV LTR U3, 5′- GACTGCTGACATCGAGCTTTCTACAAG-3′ and 5′- AAGCAGCTGCTTATATGTAGCATCTGAG-3′, the GAPDH promoter region, 5′-TACTAGCGGTTTTACGGGCG-3′ and 5′-TCGAACAGGAG-GAGCAGAGAGCGA-3′, or a portion of chromosome 12 that is not near identified open reading frames, 5′-GGCGACTTGACTTCAGA-GACAATG-3′ and 5′-GGAAAGAGGATGAGAAAGGCAGG-3′.

Resting T cell model of HIV latency

Primary CD4+ T cells were isolated by negative selection (RosetteSep, Stem Cell Technologies) from peripheral blood of healthy volunteer donors. A portion of the cells was maintained in culture (RPMI medium with 5% human AB serum) without stimulation, for 5 days. Another cell aliquot was stained with carboxyfluorescein diacetate, succinimidyl ester (CFSE, 10 μM, Molecular Probes) and held overnight. These stained cells were then infected with replication-competent NL4-3 and cultured in microplates with immobilized α-CD3 and α-CD28 antibodies to induce cell proliferation and productive HIV replication (Spina et al., 1997). After 4 days with stimulus, the CFSE-stained, infected and proliferating cells were collected and mixed with the unstimulated, unstained, and uninfected autologous CD4+ cells. The mixed co-culture was maintained for 3 days, with initial addition of exogenous rIL-2 (5 U/ml, NIH AIDS Research & Reference Reagent Program) and IL-15 (10 ng/ml, R&D Systems). On day 7,

the non-dividing CFSE-negative subpopulation ("bystander cells") was isolated by flow cytometry cell sorting using a MoFlo XDP instrument (Beckman-Coulter). The recovered resting cells, carrying non-productive latent HIV infection, were cultured in fresh medium for 2 additional days before being used in subsequent experiments (Spina et al., 2013). Maximal HIV reactivation was induced using immobilized α-CD3/α-CD28 in the presence of the integrase inhibitor raltegravir (0.1 mM). The level of virus reactivation was measured by qRT-PCR quantification of cell-associated *tat* RNA, normalized per 10^8 copies cellular 18S RNA, as described above.

Acknowledgments

We thank John Naughton for assistance preparing the manuscript, the Salk Flow Cytometry Core, the Salk Functional Genomics Core Facility, the San Diego CFAR Flow Cytometry Core (AI036214), and Grant Barish and Sunnie Yoh for technical assistance. We thank Shannon Seidel and Doug Richman for helpful discussions. This work was funded by grants from the NIH (AI072645 and AI096113), the Department of Veterans Affairs (I01BX001160), the Foundation for AIDS Research (amFAR) grant 108259-51-RGRL, a Salk-Sanofi Discovery Innovation Grant, the Nomis Foundation, the Auen Foundation, the James B. Pendleton Charitable Trust, and a fellowship from the George E. Hewitt Foundation for Medical Research (JPM).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.08.016.

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